Somatic Embryo Induction and Plantlet Regeneration of Canna × generalis from Immature Zygotic Embryo

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Abstract

Somatic embryogenesis is a unique method of in vitro regeneration, which can be used in plant reproduction, germplasm conservation, and molecular-assisted breeding. The results showed that the optimum medium for embryogenic callus induction was MS+6 mg L^{-1} 6-BA+1.5 mg L^{-1} TDZ+0.5 mg·L^{-1} NAA+30 g·L^{-1} sucrose +7 g·L^{-1} agar, and the induction rate was 47.45%. The best somatic differentiation medium was MS+2 mg·L^{-1}6-BA+1.5 mg·L^{-1} TDZ+30g·L^{-1} sucrose +7g·L^{-1} agar, and the induction rate of somatic embryos was 54.45%. The optimum medium for embryoid proliferation was MS +6mg·L^{-1} 6-BA + 1 mg·L^{-1} NAA +0.2mg·L^{-1} TDZ, and the proliferation rate and the multiplication coefficient reached 46.33% and 7.83, respectively. The mature somatic embryos were put into MS, B5, and 1/2MS medium for seedling culture. T In MS medium, true leaves grew, complete plants were obtained, and the seedling rate was 88.00%. At the same time, the survival rate of transplanting seedlings in the mixed nutrient soil with the ratio of original soil (peat: organic fertilizer: soil) =1:1:1 was as high as 98%. Cytological observation showed that the somatic embryos underwent globular, heart-shaped, torpedo, and cotyledon stages. This study established a tissue culture and regeneration system of *C. × generalis* with excellent somatic embryos, and provide basic technical support for the large-scale commercial propagation and germplasm resources protection. It will lay a foundation for further research on gene function and breeding new varieties and ideal research materials for the study of somatic embryogenesis mechanism and genetic transformation of *C. × generalis.*

Introduction

Canna spp. belongs to *the Canna* genus, Canna family, native to tropical America and Africa; also named canna lily, it is a perennial bulbous flower. It is one of the valuable ornamental plants with long-term blossoming (Mitrofanova et al. 2017) and is widely used in landscaping and purifying sewage, repairing and protecting ecological environment resulting from the advantages of strong growth, wide adaptability, easy cultivation, etc. (Dong et al. 2019; Zhang et al. 2021; Zhao et al. 2022). In addition, canna lily also has high economic value and medicinal value (Singh et al. 2019). Its flowers can be used as a natural source of anthocyanin pigments in the food ingredient industry (Srivastava and Vankar. 2010), and its roots can be used in Thai folk medicine to treat AIDS (Woradulayapinij et al. 2005). The rhizomes contain various chemical components and can treat hepatitis, stomachache, analgesia, hemostasis, and anthelmintic activity (Nirmal et al., 2007), prevent abscesses, wound healing cardiovascular and free radical-related diseases (Mahmoud et al. 2021). Currently, the propagation of the canna lily is mainly by splitting the rhizome, and breeding new *canna* cultivars rely on traditional breeding methods, which have brought many disadvantages. For example, the reproduction coefficient of conventional methods is extremely low, and long-term clonal propagation is prone to virus accumulation and variety degradation (Mishra et al. 2015). These traditional propagation methods cannot meet the commercial demand in the garden. In vitro culture of canna lily has important effects on genetic improvement and variety selection (Singh et al. 2019). Therefore, re-establishing a reliable regeneration system will lay a foundation for this plant's large-scale micropropagation and breeding engineering.

Plant tissue culture effectively produces and propagates plants rapidly and in large quantities under certain conditions. It plays an effective role in plant conservation, mass propagation, genetic improvement, and secondary metabolite production (Wang et al. 2021). Tissue culture and breeding technology have a high degree of genetic stability and can maintain the good characteristics of clonal mothers (Oh et al. 2010). In the tissue culture of the plant, there are two ways to regenerate plants in vitro. One is to obtain regenerated plants through organogenesis, and the other is to obtain regenerated plants by somatic embryogenesis. Somatic embryogenesis is a process in which a single cell develops into an embryo. In this process, somatic cells can differentiate into totipotent embryonic stem cells and then further differentiate and develop into complete plants (Horstman et al. 2017), which have the advantages of relatively stable genetic characteristics, large number, fast speed, complete structure, and high regeneration rate (Piovan et al. 2014). Somatic embryogenesis plays a vital role in plant regeneration and rapid reproduction (Tomiczak et al. 2019). The method has also been successfully applied to bananas (Shchukin. et al. 1997; Natarajan et al. 2020), lilies (Yan et al. 2020), peonies (Du et al. 2020), mango ginger (Raju et al. 2013) and other ornamental plants. Moreover, it is of great value for the large-scale propagation of superior germplasm resources and has many potential application prospects (Egertsdotter et al. 2019).

So far, the rhizomes (Wafa et al. 2016; Purshottam et al. 2019), terminal bud (Sakai and Imai. 2007), and other explants have been used to regenerate plants through organogenesis in *Canna* propagation *in vitro*. Singh et al. (2019) identified the optimal hormone concentrations for adventitious bud regeneration of *Canna* rhizomes as 6-BA 2 $mg{\cdot}L^{-1}$, TDZ 1.5 $mg{\cdot}L^{-1,}$ and IAA 0.1 $mg{\cdot}L^{-1}$, with the highest regeneration rate of 36%. Wafa et al. (2016) induced the regeneration of *Canna* plants on 6-BA 3 $mg{\cdot}L^{-1}$ and NAA 1.5 $mg{\cdot}L^{-1}$ medium, and the induction rate of adventitious buds was 73.3%, successfully establishing the regeneration system. However, the regeneration system produced by the traditional organotype method also had some defects. For example, the plants were seriously polluted in the process of culture, the browning rate was high, and the reproduction coefficient and the differentiation rate were low (Purshottam et al. 2019; Mitrofanova et al. 2017). Previous studies have reported that the leaves of *C. indica* L. were used to induce callus and regenerated plants by somatic embryogenesis. The callus was cultured in 2 $mg{\cdot}L^{-1}$ 6-BA medium for 10 to 15 days to form somatic embryos. Each culture flask produced an average of 4–5 individual cell embryos (Mishra et al. 2015). This is the only report about the canna regeneration by somatic embryogenesis; the induction rate was lower than in the other plants. Somatic embryogenesis was found to be the best method for canna cultivation, germplasm conservation, polyploidy breeding, and genetic transformation (Martinez et al. 2019). Therefore, effectively improving the rate of somatic embryo acquisition and induction efficiency was the main problem of *Canna* by SE induction. The production of SE is strongly influenced by explant sources, genotypes, culture conditions, exogenous growth regulators, and the plant growth regulators (PGRs) added to the culture medium (Kanchanapoom et al. 2011). It is expected to provide new ideas and technical references for the study of gene function and genetic improvement of *Canna*.

C. × generalis is one of the two most widely used cannas in gardens (Bailey, 1923) and is an important material for improving canna cultivars (Li. 2022). The purpose of this study is to investigate the effects of different plant growth regulators, medium types, and culture conditions during the process of induction and germination of SE from the immature embryo of *C. × generalis*. Establish an efficient system for somatic embryo induction and regeneration and make clear the stages and the structural characteristics of SE formation and development by histomorphological observation. This study will provide an effective regeneration method for genetic improvement, germplasm innovation, and a new variety selection of *C. × generalis*.

Materials and methods

Plant material

After anthesis for 30 days, the immature fruit was taken from the germplasm resources garden of the canna lily at Guizhou University from July to October 2021. First, the fruits were washed with liquid detergent for 10 minutes and rinsed with distilled water 5 times. Next, the immature seeds were taken from the fruits on the ultra-clean workbench and placed in a beaker. Then the fruits were treated with 75% ethanol for 30 s, rinsed 5–6 times using sterile distilled water, disinfected with 0.1% *(w/v)* mercuric chloride for 7 min, rinsed 3–5 times with sterile distilled water, and dried on a sterile filter paper. After that, the immature zygotic embryo were isolated from intact seeds for embryogenic callus induction.

Embryogenic callus induction

MS medium was added with 30 g L^{-1} sucrose, and 7 g L^{-1} agar was used as the basic media (pH 5.8). Cultivation was conducted with different concentration plant growth regulators, namely, 6-BA (2.0, 4.0, 6.0 mg L^{-1}), NAA (0.1, 0.2, 0.5 mg L^{-1}), and TDZ (1.0, 1.5, 2.0 mg L^{-1}). Nine treatments were selected for the three-factor and three-level orthogonal experimental design. The sterilized young embryo was a 0.5 mm thin slice with 30 explants per treatment, replicated three times. In the second week after inoculation, the explants were transferred to a fresh induction medium daily and then transferred to a fresh medium every two weeks. The growth of embryogenic callus was observed weekly, and embryogenic callus induction was counted for 30 days respectively.

On the 15th day of induction, two kinds of calli of different shapes and forms were selected to observe the occurrence of embryonic callus microscopically. First, the MS medium on the surface was removed by gently washing it with sterile distilled water. The samples were then sucked dry, observed, and photographed with an optical microscope (DM 750 LED), and the embryonic callus with distinct characteristics was selected for paraffin section observation.

$$\text{Embryogenic callus induction rate}(\%) = \frac{\text{number of explants induced to obtain embryogenic callus}}{\text{total number of inoculated explants}} \times 100\%$$

Somatic Embryogenesis Induction

Six treatments by orthogonal experimental design were set up through supplement with 6-BA (0.0, 1.0, 1.5, 2.0, 2.5, 3 mg L^{-1}) and TDZ (0.0, 0.5, 1, 1.5, 2, 2.5 mg L^{-1}). The embryogenic callus with the same growth condition was cut into 0.5 cm × 0.5 cm in size and inoculated on the medium, 10 bottles for each treatment, replicated three times. The growth status of embryoid induction was counted for 30 days, respectively.

In order to observe somatic embryogenesis microscopically, samples were collected from MS medium. When the callus showed obvious somatic embryo characteristics, samples of different somatic embryo development stages induced by embryogenic callus were taken every 5 days and then gently washed with sterile distilled water to remove MS medium on the surface. The samples were then sucked dry, viewed, and photographed using a light microscope (LEICA ICC50W).

$$\text{Somatic embryogenesis rate}\,(\%) = \frac{\text{number of calli producing somatic embryos}}{\text{number of explants inoculated with embryogenic calli}} \times 100\,\%$$

Somatic embryo proliferation culture

The embryoids with the same growth after subculture were inoculated on the embryoid proliferation medium with different combinations of plant growth regulators. The experiments were carried out with 6-BA (1.0, 3.0, 6.0 mg L^{-1}), NAA (1.0, 1.5 mg L^{-1}), and TDZ (0.2, 0.5 mg L^{-1}), respectively. Each treatment of 10 bottles was replicated three times, and the growth status of embryoid proliferation was counted for 30 days.

Somatic embryo germination and conversion

The obtained white or green normal somatic embryos with a diameter of 1 cm and regular shape were inoculated onto the designed somatic embryo maturation medium (Table 4) to induce somatic embryo maturation. First, 10 bottles of each medium formula were inoculated, each treated material was inoculated with 25 individual cell embryos, and each treatment was replicated three times. Next, the embryogenesis of somatic cells was measured after 15 days of culture, then inoculating the mature somatic embryos into a designed somatic embryo germination culture medium, inducing the mature somatic embryos to regenerate, observing and counting the plant regeneration condition after 30 days.

Table 3
Effect of plant growth regulators (PGRs) on the Somatic Embryo Proliferation from *Canna × generalis*

Concentrations of PGRs (mg/L)			Somatic embryo proliferation rate (%)	Proliferation times
6-BA	NAA	TDZ		
1	1	0.2	0.00 ± 0.00d	0.00 ± 0.00d
1	1.5	0.5	0.00 ± 0.00d	0.00 ± 0.00d
3	1	0.2	14.00 ± 2.00c	0.12 ± 0.03d
3	1.5	0.5	34.33 ± 2.96b	2.99 ± 0.18c
6	1	0.2	46.33 ± 2.03a	7.83 ± 0.16a
6	1.5	0.5	37.33 ± 2.96b	5.53 ± 0.13b

*Data in the table represent mean ± standard error, and different letters (a, b,…) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test)

Table 4
Effects of different medium formulations on seedling culture of *Canna × generalis* embryoids

culture medium	Number of embryos	Germination rate (%)	Seedling rate (%)	Seedling growth status
MS	25	94.67 ± 3.53a	88.00 ± 4.62a	green and grow robustly
B5	20	23.33 ± 1.66c	13.33 ± 1.67b	green and grow weakly
1/2MS	22	52.66 ± 4.09b	24.00 ± 7.62b	green and grow robustly

*Data in the table represent mean ± standard error, and different letters (a, b,…) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test)

Acclimation and transplantation

Vigorous embryoid sterile seedlings were selected and moved to the greenhouse for 1 day to adapt to the greenhouse environment. Next, the seedlings were uncovered and refined for 2 days. Then the germinated seedlings were transplanted to the peat screened by the research group in the early stage: organic fertilizer: soil = 1:1:1 for strong seedling culture.

$$\text{Germination rate}(\%) = \frac{\text{Number of germinated somatic embryos}}{\text{Number of somatic embryos inoculated}} \times 100\,\%$$

$$\text{Survival rate}(\%) = \frac{\text{No. of surviving plants}}{\text{No. of exflasked plants}} \times 100\,\%$$

Histomorphology observation

Two kinds of calli of different shapes and somatic embryos of different stages of good growth were fixed overnight with 75% FAA (formaldehyde, glacial acetic acid, 75% ethanol 1:1:18). After discarding the fixing solution, the somatic embryos were treated with 70, 80, 95 and 100% ethanol successively, and soaked in anhydrous ethanol twice to ensure complete dehydration. It is then treated with xylene for transparency. After paraffin embedding, the wax blocks were divided according to the material placement position, and the divided wax blocks were trimmed into trapezoids. It was placed at 4°C for 24 h, and paraffin sections were prepared. The embedded wax blocks were cut into 8–10 μm slices using a microtome (Erma, Japan), and the slides were dried in a slide dryer at 38 °C. The sections were observed and photographed under an optical microscope (LEICA ICC50W) after dewaxing, ethanol remixing, zorubin green staining, and neutral gum seal. The different stages of SE were recorded, and the dynamic changes in cells and their origin during SE were observed.

Culture conditions

The basic medium used in this experiment was MS medium, and the reagents were purchased from Beijing Solarbio Biological Company. The medium pH of all experiments was 5.8 ± 0.2, sterilized at 121°C for 25 minutes; unless otherwise specified, MS solid medium was supplemented with 7 $g{\cdot}L^{-1}$ agar as a coagulant and 30 $g{\cdot}L^{-1}$ sucrose. All plants were cultured under a light intensity of 25–35 μ $mol{\cdot}m^{-2}{\cdot}s^{-1}$ at room temperature of (25 ± 2) °C.

Statistical analysis

Excel software (Excel 2017) was used for statistical data. SPSS software (SPSS 20.0) was used for data processing and one-way analysis of variance (ANOVA), and Duncan's multiple tests was performed. All test treatments were set with three biological replicates.

Results And Discussion

Results and discussions

The effects of plant growth regulators on embryogenic callus induction

Obtaining embryogenic callus was the key to somatic embryo induction (Li et al. 2021), and plant growth regulation significantly affected callus induction (Zhao et al. 2011; Zhou et al. 2012). Similar to previous studies, this study found that the immature embryo began to expand and produce two kinds of white granular callus and white massive callus on the 7th day. Both calli can proliferate, but the white massive type cannot be used for later differentiation. It was inferred that the white granular was an embryogenic callus (Fig. 1B). The white massive type was a non-embryogenic callus (Fig. 1C). Through the paraffin section, it was observed that the embryogenic callus cells were small and closely arranged. There are few vacuoles, dense cytoplasm, and a true nucleus (Fig. 1E), while the non-embryogenic callus is the opposite, with no nucleus found (Fig. 1F).

In callus induction, MS + 6 mg·L-16-BA + 1.5 mg·L^{-1} TDZ + 0.5 mg·L^{-1} NAA had a callus induction rate of 76.33%, of which the embryogenic callus induction rate was 47.45%. The non-embryogenic callus induction rate was 28.88%, which was significantly higher than other combinations (Table 1). Under the same formula, the different types of calli mainly depend on the way of induction and the physiological state of plants. In this study, there are two types of calli in the same formula, which may be the result of the cutting method and the physiological state of plants during the operation. With 2 mg · L^{-1}6-BA, the induction rate of callus was 0%, which increased with the increase of 6-BA and NAA concentrations. When TDZ exceeded the optimal concentration, the callus induction rate decreased, possibly due to the induction of plant growth regulators on the callus, low-concentration induction, and high-concentration inhibition. In addition, it was found that after 5–6 subcultures of embryogenic calli, the embryogenicity of embryogenic calli became weaker, similar to the results of previous studies that other plant calli of Myrica rubra did not maintain embryogenic ability in long-term culture (Martins et al. 2022). To sum up, MS + 6 mg·L^{-1} 6-BA + 1.5 mg·L^{-1} TDZ + 0.5 mg·L^{-1} NAA is the best medium combination for inducing embryogenic calli from the young embryo of *C. × generalis* in this study.

Table 1
Effect of plant growth regulators (PGRs) on the Embryogenic callus induction from *Canna × generalis*

Concentrations of PGRs (mg/L)			Non-embryonic callus induction rate (%)	Embryogenic callus induction rate (%)	Callus formation rate (%)
6-BA	NAA	TDZ			
2	0.1	1	0 ± 0g	0 ± 0g	0 ± 0g
2	0.2	1.5	0 ± 0g	0 ± 0g	0 ± 0g
2	0.5	2	0 ± 0g	0 ± 0g	0 ± 0g
4	0.1	1.5	7.29 ± 0.21f	9.00 ± 1.19e	16.29 ± 0.31f
4	0.2	2	9.61 ± 0.06e	17.01 ± 0.89d	26.62 ± 0.02e
4	0.5	1	10.33 ± 1.02d	23.38 ± 1.75c	34.16 ± 1.36d
6	0.1	2	27.50 ± 1.90c	26.33 ± 0.88bc	53.83 ± 1.96c
6	0.2	1	36.33 ± 1.88b	30.00 ± 1.15b	66.33 ± 1.89b
6	0.5	1.5	28.88 ± 0.86a	47.45 ± 3.72a	76.33 ± 0.88a

*Data in the table represent mean ± standard error, and different letters (a, b,…) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test)

Induction of somatic embryogenesis

Plant growth regulators were generally the most critical factors in somatic embryo induction and development (Feheir et al. 2003; Shekhawat et al. 2021). The most commonly used are 2,4-D, IBA, GA_3 and 6-BA (Stasolla and Yeung 2003; Miroshnichenko et al. 2017). Cytokinins are essential for somatic embryogenesis (Pereira et al. 2020), and 6-BA has a certain promotion effect on somatic cell embryogenesis (Martins et al. 2022). In this study, we tried different cytokinin combinations for somatic embryogenesis. The number of somatic embryos induced on MS + 2 mg L^{-1} 6-BA + 1.5 mg L^{-1} TDZ + 30 g L^{-1} sucrose + 7 g L^{-1} agar was the highest, and the induction rate of somatic embryos was 54.45% (Table 2). On average, 10–15 somatic embryos produced per 0.5 g embryogenic callus were in good condition, showing white at the early stage and then light green or dark green (Fig. 2), and 4–5 embryos were found to be higher than previous research results (Mishra et al. 2015). The induced somatic embryo can be subcultured 5 times, and the activity of the somatic embryo can be reduced along with the increase of the subculture times to the sixth time. The decrease of 5–6 cells suggests that the decrease in regeneration ability may result from the gradual aging of plant cells and the decrease of totipotency (Murashig et al. 1965).

Table 2
Effect of plant growth regulators (PGRs) on the Somatic Embryo Induction from *Canna × generalis*

Concentrations of PGRs (mg/L)		Induction rate of somatic embryo (%)
6-BA	TDZ	
0	0	10.88 ± 1.15e
1	0.5	23.33 ± 1.92d
1.5	1	39.40.67 ± 1.93b
2	1.5	54.45 ± 2.93a
2.5	2	47.77 ± 1.11a
3	2.5	32.22 ± 1.11c
*Data in the table represent mean ± standard error, and different letters (a, b,…) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test)		

Morphological and histological studies were still crucial in the induction of plant embryogenic callus and somatic embryos (Islam et al. 2013). Previous studies found that embryogenesis and non-embryogenesis callus can be distinguished by tissue morphology (Maadon et al. 2016), and dynamic changes of cells at different stages of somatic embryogenesis can be observed (Ji et al. 2017). In this study, the observation results of the C. × generalis paraffin section showed the regeneration mode of *C. × generalis* in somatic embryogenesis type (Fig. 3). During development, embryogenic calli developed into spherical embryos (Fig. 3a, e), heart-shaped embryos (Fig. 3b, f), torpedo embryos (Fig. 3c, g) and cotyledon embryos (Fig. 3d, h), with no vascular tissue connection to the parent. In the longitudinal section of the cotyledon embryo at the early stage of differentiation, it is evident that there are stem tip meristems and root meristems at the top and base, respectively. In the early stage of somatic embryo formation, there are apparent two-level embryogenic cell mass structures with prominent bipolar characteristics.

This study found that after two weeks of subculture on a culture medium, somatic embryos and embryogenic calli developed into spherical embryos, heart-shaped embryos, torpedo embryos, and cotyledon embryos, which were similar to previous studies (Yang and Zhang 2010). In addition, previous studies showed that heart-shaped embryos were typical characteristics of dicotyledonous plant cell embryos (Kurczynska et al. 2007), and heart-shaped embryos were also observed during monocotyledonous plant SE, Such as lily olved from extinct dicotyledonous plants (Henllo 1911). The existence of an SE heart-shaped embryo of *C. × generalis* grandiflora provides strong evidence for this speculation. At the same time, this study was similar to other research results of somatic embryogenesis and embryo maturation (Fang et al. 2022; Yang and Zhang 2010; Vahdati et al. 2008). Moreover, they

have no vascular tissue connection with the maternal body, which was consistent with previous research and observation results (Zou et al. 2019; Quinga et al. 2018).

Somatic embryo proliferation culture

It can be seen from Table 15 that with MS medium as the basic medium, the somatic embryos with the same growth momentum are inoculated into the proliferation medium. When the 6-BA concentration is 1 $mg{\cdot}L^{-1}$, there is no embryoid proliferation. When the 6-BA concentration is 3-6$mg{\cdot}L^{-1}$, the somatic cell embryos produce secondary embryo proliferation. But when the 6-BA concentration is 6 $mg{\cdot}L^{-1}$, NAA concentration is 1 $mg{\cdot}L^{-1}$, NAA concentration is 0.2 $mg{\cdot}L^{-1}$, the proliferation rate is 46.33%, and the proliferation coefficient can reach 7.83, which is significantly higher than other combinations, consistent with the previous research results(Mishra et al. 2015).

When the concentration of cytokinin 6-BA was 3 $mg{\cdot}L^{-1}$, the proliferation of somatic embryos was white (Fig. 4B), and the generation of secondary embryos was earlier than 6 $mg{\cdot}L^{-1}$5 d when the concentration of 6-BA was 6 $mg{\cdot}L^{-1}$. When the concentration of 6-BA was 6 $mg{\cdot}L^{-1}$, the somatic embryos were good, and the color was dark green. The secondary embryos were white or light green (Fig. 4B, C), similar to the results of secondary embryogenesis induced by a higher concentration of 6-BA (Soundar Raju et al. 2013). In addition, some somatic embryos did not proliferate under low concentrations of cell division, and somatic embryo maturation occurred (Fig. 4D). In conclusion, MS + 6 $mg{\cdot}L^{-1}$ 6-BA + 1 $mg{\cdot}L^{-1}$ NAA + 0.2 $mg{\cdot}L^{-1}$TDZ was the best formula for somatic embryos, which could provide technical reference for the optimization of later somatic embryo proliferation.

Somatic embryo germination and conversion

It can be seen from Tables 4 and 5 that the mature embryoid developed into a plant in several culture media. The germination and seedling rates of MS culture were 94.67% and 88.00%, respectively, which were significantly higher than B5 and 1/2MS culture media. In MS, part of the embryoid has a proliferation phenomenon of a secondary embryo. However, the germination and seedling rates in B5 were 23.33% and 13.33%, respectively, and the seedling rate was relatively low, so the effect was not ideal. Therefore, the medium suitable for embryoid germination and seedling formation was MS, similar to the previous study that it was beneficial to developing SE in a hormone-free medium or low hormone concentration for chestnut (Lu et al., 2017). The reasons may be because high hormone concentrations inhibit SE tissue differentiation and interfere with the normal development of the apical meristem (Halperin and Wetherll 1964).

As depicted in Fig. 5, it was observed that embryogenic calli induce somatic embryos and germinate into complete plants. In this study, the mature somatic embryos were inoculated into the MS medium (Fig. 5A). When the somatic embryos were inoculated into the MS medium for 15 days, the plants began to see the root and bud germinating and growing at the same time (Fig. 5B). After 20 days, the plants

grew luxuriantly. The leaves were green (Fig. 5C, D), which could induce robust small plants (Fig. 5E). The medium used at this stage does not contain growth regulators, which proves to be conducive to the germination of somatic embryos, with well-developed roots and buds and the complete plants formed (Fig. 5E). The results were similar to other plants that have also observed the germination of somatic embryos in MS medium without regulators and the development of complete plants (Ferreira et al. 2022). In addition, somatic embryos with bipolar nature can directly develop into complete plantlets without the rooting stage (Arnold et al. 2002), which can shorten the seedling period and save costs.

Acclimation and transplantation

From Fig. 6, it was observed that embryoid test tube seedlings were obtained for strong seedling culture(Fig. 6-A), opened the bottle and domesticated (Fig. 6-B), and directly transplanted the domesticated seedlings into a greenhouse pot with a volume ratio of nutrient soil (peat: organic fertilizer: soil = 1:1:1) for culture. After 7 days, it was observed that *C. × generalis* plantain started to grow new green leaves in good growth condition (Fig. 6-C). After 15 days, the seedlings developed into normal healthy small plants and grew vigorously with dark green leaves (Fig. 6-D). The results showed that in the process of domestication and transplanting, the addition of seedling substrate and peat provided nutrients for its growth and development, and the regeneration rate of *C. × generalis* was 98% higher than the previous research (Mishra et al., 2015). Therefore, the mixed substrate with a volume ratio of peat: organic fertilizer: soil = 1:1:1 was the best cultivation substrate for *C. × generalis*.

Conclusions

This study uses young embryos as materials, and the regeneration system of *C. × generalis* was successfully established through cell embryogenesis. It took about 10 weeks to obtain complete small plants. In addition, microscopic and histological analysis showed that somatic embryogenesis belongs to the indirect type. Typical morphogenesis periods such as globular, heart-shaped, torpedo-shaped, and cotyledon-shaped were observed during the development of somatic embryos. These results would help broaden our understanding of the origin and development of somatic embryos. The establishment of the regeneration system is conducive to transgenic and genome editing technology and promotes the genetic improvement and breeding process of *C. × generalis* grandiflora. Furthermore, it can provide reference for somatic embryo culture of other varieties of *C. × generalis*, accelerate the rapid application of molecular breeding, and lay a foundation for breeding new varieties of *C. × generalis*.

Declarations

Author Contributions: All authors contributed to the study conception. Material preparation was performed by ZYG, FY and GFW. Data collection was accomplished by ZYG and MLS. Data analysis,

experiment design and supervision were performed by XJP, FY and WEZ. The draft of the manuscript was written by ZYG and WEZ revised the article. All authors commented on previous versions of the manuscript and all authors read and approved the final manuscript.

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Figures

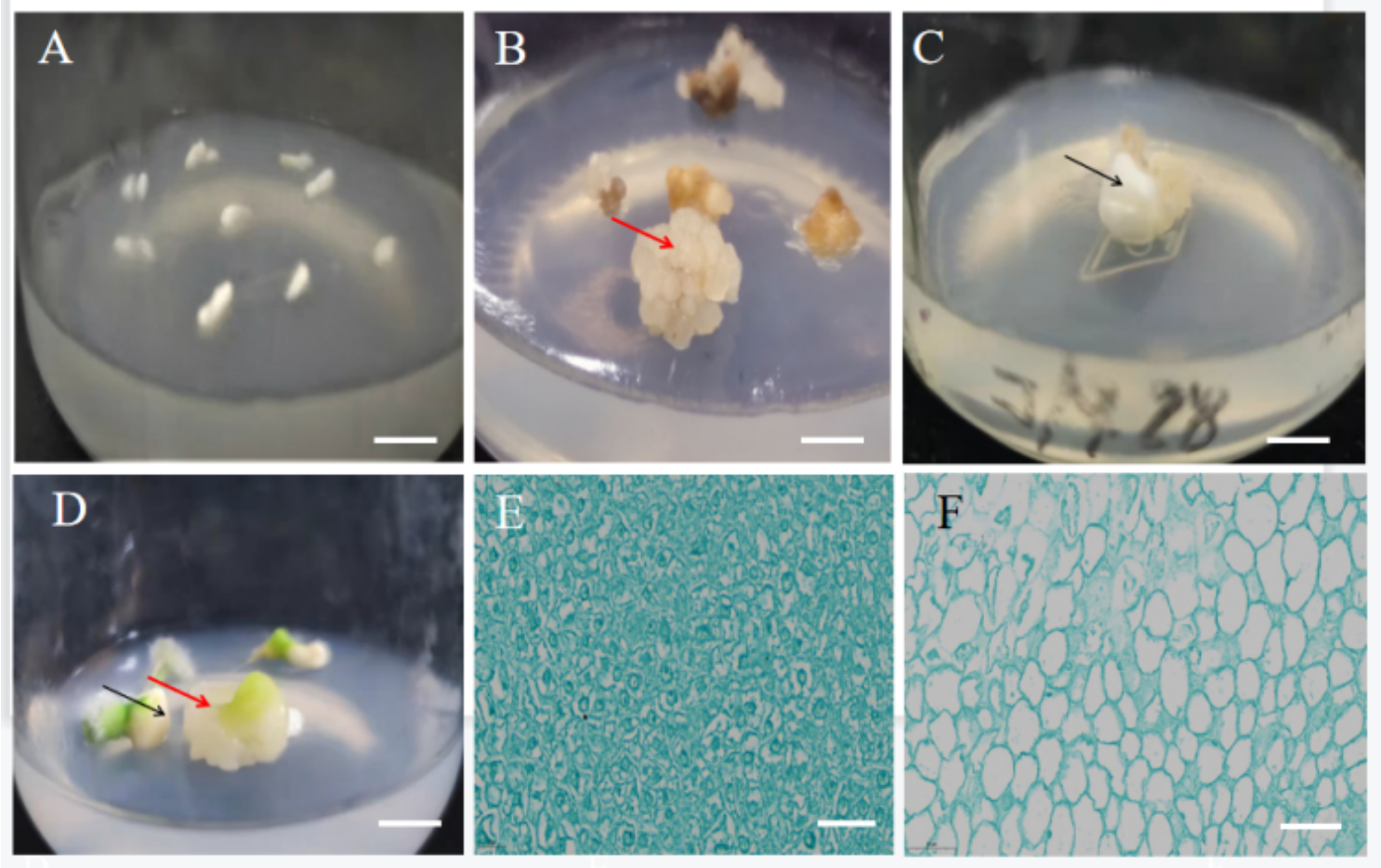

Figure 1

Callus induction from immature embryo o*f C. × generalis*

a. Explant status after inoculation for 0 days. **b.** White granular embryogenic calli after 15 days inoculation. **c.** White massive non-embryonic callus after 15 days inoculation. **d.** Embryogenic calli and non-embryonic callus after 15 days inoculation. (The red arrow indicates embryogenic callus, and the black arrow indicates non-embryogenic callus). **e.** Cell morphology of embryogenic calli. **f.** Cell morphology of non-embryogenic callus. Bars: 2 cm: (a-d); 50um: (e-f)

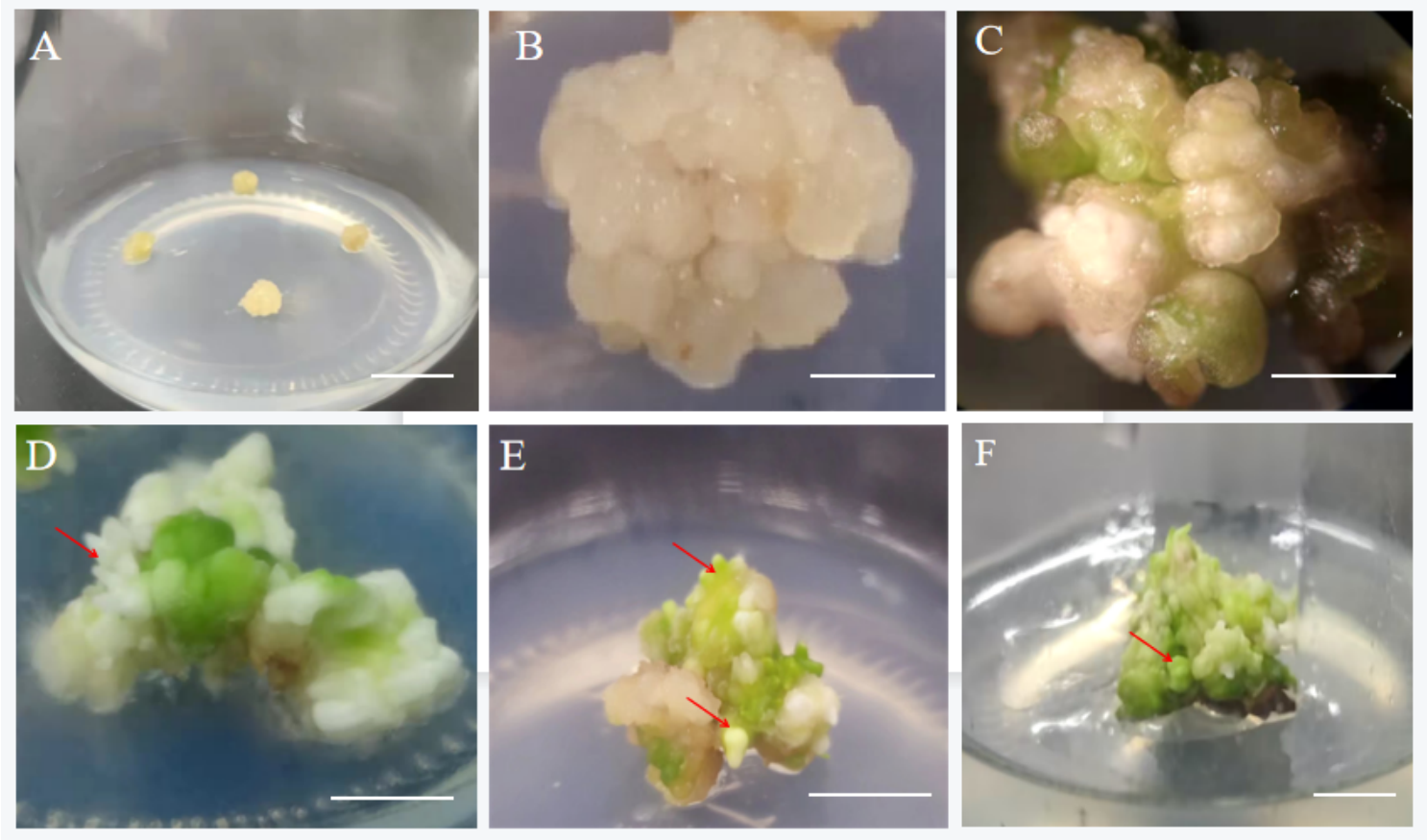

Figure 2

Somatic induction of *C. × generalis*

a. Embryogenic callus were inoculated at 0d (bar = 2 cm) .**b.** Embryogenic callus (bar = 5mm).**c.** Pre-embryonic culture (bar = 5mm).**d.** Secondary somatic embryos (SSE) produced from primary somatic embryos after 20 days culture (bar = 5mm).**e,f.** Secondary somatic embryos (SSE) produced from primary somatic embryos after 25 days culture, (bar = 2cm). The red arrow in the figure shows somatic embryos at different stages

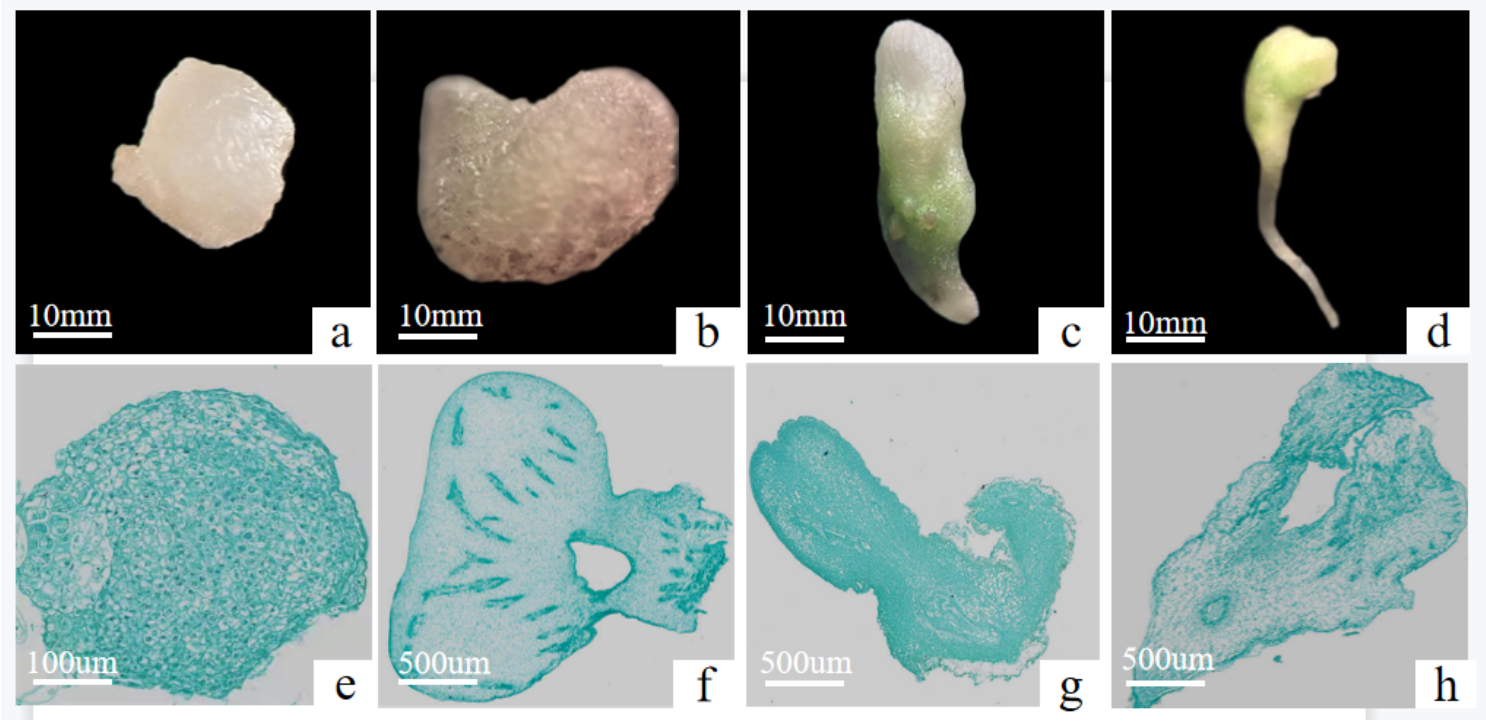

Figure 3

Phenotype (a-d) and microscopic observation (e-h) of four developmental stages of somatic embryogenesis

a, e globular embryo. **b, f** Heart-shaped embryo. **c, g** Fish-shaped embryo**. d, h** cotyledon embryo.

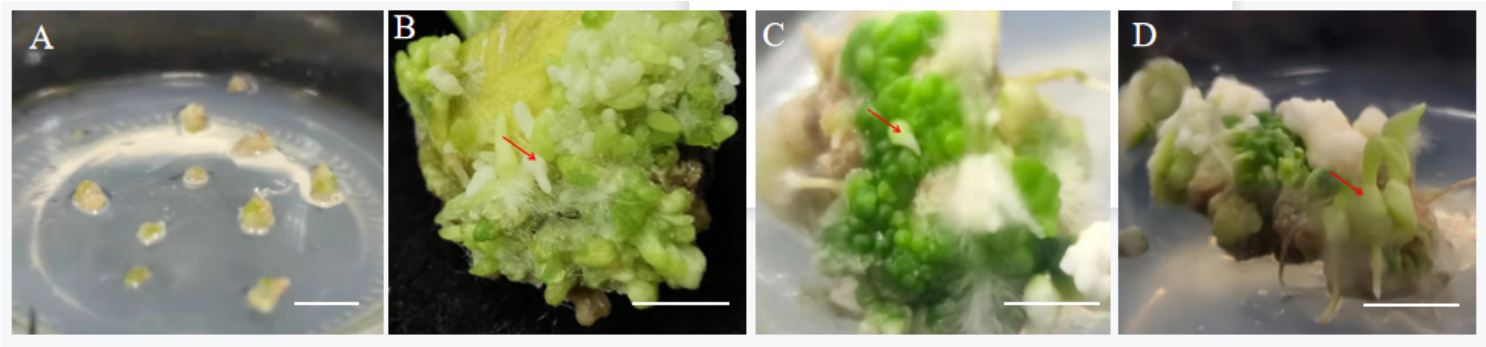

Figure 4

Proliferation of somatic embryoids under different culture days

a. roliferation and growth of somatic embryos after inoculation for 0 days. b,c. Proliferation and growth of secondary embryos inoculated for 20 days. **d.** Proliferation and growth of secondary embryos inoculated for 25 days. Note: Bar: 1 cm

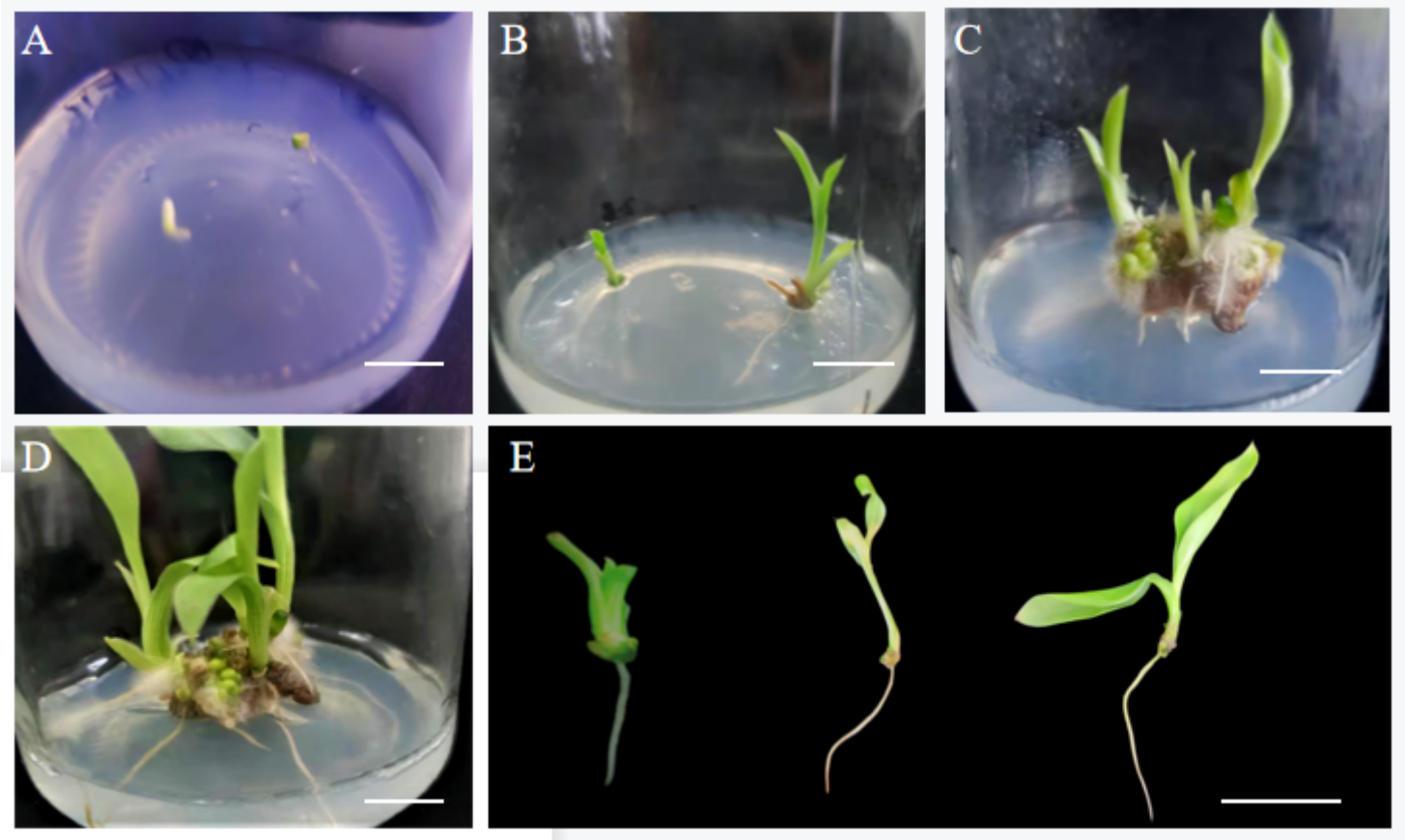

Figure 5

Somatic embryo germination and plant regeneration of *C. × generalis*

a. Somatic embryo inoculated on the germination media.**b.** Seedling status after germinating for 15d. **c.** Seedling status after germinating for 20d somatic embryo germination and seedling status**. d.** Seedling status after germinating for 25d. **e.** Germination process of Somatic embryo. Bars: 2 cm: (a-d); 5cm :(e).

Figure 6

Transplantation of somatic embryo seedlings of *C. × generalis*

a. Regenerated somatic seedlings. **b.** Domestication and transplanting of seedlings with open cover. **c.** The growth state of somatic embryo plantlets transplanted for 7 days. **d.** Growth state of somatic embryo plantlets transplanted for 15 days. Bar: 2 cm.